Gabapentin inhibits catecholamine release from adrenal chromaffin cells

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Abstract

Background—Gabapentin is most commonly prescribed for chronic pain, but acute perioperative effects including preemptive analgesia and hemodynamic stabilization have also been reported. Adrenal chromaffin cells are a widely used model to investigate neurosecretion and adrenal catecholamines play important physiological roles and contribute to the acute stress response. However, the effects of gabapentin on adrenal catecholamine release have never been tested.

Methods—Primary cultures of bovine adrenal chromaffin cells were treated with gabapentin or vehicle for 18–24 h. We quantified catecholamine secretion from dishes of cells using high performance liquid chromatography, and resolved exocytosis of individual secretory vesicles from single cells using carbon fiber amperometry. Voltage-gated calcium channel currents (I_{Ca}) were recorded using patch-clamp electrophysiology, and intracellular $[Ca^{2+}]$ using fluorescent imaging.

Results—Gabapentin produced statistically significant reductions in catecholamine secretion evoked by cholinergic agonists (24 ± 3 % n = 12) or KCl (16 ± 4 % n = 8) (mean $\pm$ SEM) but did not inhibit Ca^{2+} entry or I_{Ca}. Amperometry (n = 51 cells) revealed that gabapentin inhibited the number of vesicles released upon stimulation, with no change in quantal size or kinetics of these unitary events.

Conclusions—We show Ca^{2+} entry was not inhibited by gabapentin, but was less effective at triggering vesicle fusion. Our work also demonstrates that chromaffin cells are a useful model to further investigate the cellular mechanism(s) by which gabapentin controls neurosecretion. Moreover, it identifies altered adrenal catecholamine release as a potential contributor to some of the beneficial perioperative effects of gabapentin.

INTRODUCTION

Gabapentin is most commonly prescribed for chronic pain states but there is increasing interest in potentially beneficial effects of perioperative dosing. For example, several randomized control trials indicate that gabapentin has acute preemptive analgesic effects on intraoperative and postoperative pain[1]. Preoperative gabapentin also blunted the hemodynamic response to direct laryngoscopy and tracheal intubation [2,3]. Although the underlying mechanism(s) remain unclear, previous clinical studies have shown these procedures stimulate a sympathoadrenal stress response evidenced by increases in circulating catecholamine levels [4–7]

While it is clear that gabapentin has a constellation of analgesic, antihyperalgesic, and perioperative hemodynamic effects, the cellular mechanism(s) that underlie these actions are not well understood. Although it was originally developed as a mimetic of the inhibitory neurotransmitter γ-aminobutyric acid, the consensus now holds that gabapentin exerts little effect on γ-aminobutyric acid signaling, but rather binds with high affinity to the $\alpha_2\delta$ subunit of voltage-gated calcium channels (Ca^{2+}-channels) [8]. Calcium influx through Ca^{2+} channels triggers exocytosis of secretory vesicles, leading to the hypothesis that gabapentin reduces transmitter release by reducing Ca^{2+} entry. Indeed, some studies reported acute inhibition of Ca^{2+} channel currents (I_{Ca}) by gabapentin or pregabalin [9–12], although others found no effect [13–15]. Perhaps of more relevance to the perioperative setting, longer-term (17–48 h) incubation with gabapentin / pregabalin reduces the amplitude of I_{Ca} by disrupting trafficking which results in fewer channels at the plasma membrane [13,14,16,17]. Gabapentin might also inhibit neurotransmitter release by other mechanisms. Consistent with this, spontaneous synaptic release events or exocytosis evoked by hypertonic sucrose were inhibited by gabapentin / pregabalin, even though these events are thought to occur independent from Ca^{2+} entry [18,19]. Thus, regulation of neurotransmitter and hormone release by gabapentin is likely complex and might involve effects on the transmitter release machinery in addition to altered Ca^{2+} entry and/or altered excitability in neuronal circuits.

The rationale for our study, the first to investigate the effects of gabapentin on adrenal chromaffin cells, was twofold. First, chromaffin cells are widely used to investigate neurosecretion [20–22], so provide a good model to evaluate the cellular mechanisms of gabapentin. Second, chromaffin cells release a complex cocktail of catecholamines, endogenous opioids [23] and other transmitters that play central roles in the acute stress response and possibly in stress-related enhancement of mechanical hyperalgesia [24]. Therefore, we postulated that perioperative hemodynamic stabilization, and perhaps other stress-related effects of gabapentin, might be partly mediated through reduced catecholamine release.

MATERIALS AND METHODS

Cell culture

Adult bovine adrenal glands were obtained from a local slaughterhouse and chromaffin cells were prepared by digestion with collagenase followed by density gradient centrifugation as described previously[25]. The cells were plated onto coverslips coated with collagen (at a density of 0.3–0.4 x 10^6 cells/mL for $[Ca^{2+}]_i$ measurements or ~0.2 x 10^6 cell /mL for patch

clamp recordings). For secretion studies cells were plated in 24-well tissue culture plates at a density of ~ 0.3×10^6 cells per well. Fibroblasts were effectively suppressed with cytosine arabinoside (10 μM) (Sigma-Aldrich; St Louis MO), leaving relatively pure chromaffin cell cultures. The culture medium consisted of Dulbecco's modified Eagle medium \ F12 (1:1) supplemented with fetal bovine serum (10%), glutamine (2 mM), penicillin/streptomycin (100 unit mL^{-1}/100 μg mL^{-1}), cytosine arabinoside (10 μM) and 5-fluorodeoxyuridine (10 μM). Unless noted otherwise, all tissue culture reagents were from Invitrogen (Carlsbad, CA) apart from fetal bovine serum which was from Hyclone (Logan, UT). All experiments were performed 2–5 days following cell isolation.

$[Ca^{2+}]_i$ Measurements

Free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured in cells loaded with the fluorescent Ca^{2+} indicator Fura-2 (Molecular Probes, Eugene OR) using an InCyt IM2 fluorescence imaging system (Intracellular Imaging Inc., Cincinnati, OH) as described previously [25]. Cells were washed twice with HEPES-buffered Hanks Balanced Salt Solution (Invitrogen) and incubated for 30–45 min with 3 μM Fura-2 AM at 37°C. Cells were then washed in Fura-free solution for 30–60 min before recording. For recording, the coverslip with the cells attached was transferred to a recording chamber and mounted on the stage of a Nikon TE2000 fluorescence microscope (Nikon Instruments Inc., Melville, NY). The recording chamber had a volume of ~300–400 μL and was continually perfused with fresh solution from gravity-fed reservoirs at a flow rate of ~4 ml/min. The extracellular solution comprised (in mM): 136 NaCl, 2 KCl, 1 MgCl, 10 Glucose, 10 HEPES, 2 CaCl, pH 7.3 osmolarity ~ 305 mOsm. Cells were alternately excited at wavelengths of 340 nm and 380 nm and emission at 510 nm detected using a pixelfly digital camera. The ratio of fluorescence at 340 nm/380 nm excitation was collected every 2 s throughout the experiment and converted to $[Ca^{2+}]_i$ using an *in vitro* calibration curve generated by adding 15.8 μM Fura-2 pentapotassium salt to solutions containing 1 mM $MgCl_2$ and known concentrations of Ca^{2+} (0 1350 nM). Data analysis was performed using OriginPro software (Originlab Corporation, Northampton, MA).

Catecholamine release experiments

Cells in 24-well plates were washed twice with extracellular solution and incubated in this solution for 30 mins at ~37 °C. For gabapentin (Tocris Cookson, Ellisville, MO, or Ascent Scientific, Princeton, NJ) treated cells, the drug was also present in all solutions throughout the experiment. The solution was then removed and replaced with fresh solution to determine basal release or solution containing 100 μM carbachol (Sigma-Aldrich) to stimulate secretion. Alternatively, cells were stimulated using the selective nicotinic receptor agonist 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) (30 μM) (Sigma-Aldrich) or a solution with elevated (30 mM) KCl. After the 5-min stimulation period at ~37 °C the cells were placed on ice and the solution collected and added to an equal volume of ice-cold 0.4 M perchloric acid. Perchloric acid was also added to the tissue culture wells to lyse the cells and extract the non-released catecholamines. The catecholamine content of the samples was determined by a specific high performance liquid chromatography (HPLC) assay utilizing an Antec Decade (oxidation potential: 0.7 V) electrochemical detector in the Neurochemistry Core of the Center for Molecular Neuroscience at Vanderbilt University. Samples (20 μL) were injected using a Waters 717+ autosampler onto a Kinetex 2.6u C18 100A 100x4.6mm HPLC column (Phenomenex, Torrance, CA) and biogenic amines eluted with a mobile phase consisting of 89.5% 0.1 M TCA, 10^{-2} M sodium acetate, 10^{-4} M EDTA and 10.5 % methanol (pH 3.8). The amount of catecholamine released into the bathing medium during the 5-min stimulation period was expressed as a percentage of the total catecholamine content for that dish of cells. Each plate consisted of control and gabapentin treated conditions performed in duplicate. Duplicates for each condition were averaged and

compared. Personnel performing the catecholamine detection were blinded to which samples were from control or gabapentin treated cells. Each dataset were obtained from several independent experiments performed on different plates of cells (n is indicated in text). Each dataset was also performed on several different preparations of cells (*i.e.*, cells isolated from different animals). To calculate an EC_{50} for inhibition of catecholamine secretion, data from experiments using different concentrations of gabapentin were plotted and fit with a Boltzmann function of the form: $Y = Ymax / (1 + 10^{\wedge}(Log\ EC_{50} - X))$; where Y = percent inhibition of secretion and X is the concentration of gabapentin. The Hill slope was assumed to be 1 and the curve was fit with the least squares method in Prism5 software (GraphPad Software Inc., San Diego, CA).

Patch-clamp electrophysiology

Electrodes were pulled from borosilicate glass capillary tubes (World Precision Instruments, Sarasota, FL), coated with dental wax (Electron Microscopy Sciences, Hatfield, PA) and fire polished to a final resistance of ~2 MΩ when filled with a CsCl-based internal solution. Cells were voltage-clamped in the conventional whole-cell configuration using an Axopatch 200B amplifier, Digidata 1400A interface, and PClamp10 (Clampex) acquisition software (Molecular Devices, Sunnyvale, CA). Analog data were filtered at 2 kHz and digitized at 20 μs/point (50 kHz). Series resistance was partially compensated using the Axopatch circuitry and data for I_{Ca} were subjected to linear capacitance and leak subtraction using standard pulse / number protocols. Cell membrane capacitance was determined for each cell using the membrane test protocol in Clampex software. I_{Ca} were activated by brief 20 ms step depolarizations to a predetermined peak (10–30 mV) from a holding potential of −80 mV. To assess inactivation, 500 ms voltage step commends were used as detailed in the results section. Data were analyzed using PClamp10 (Clampfit), OriginPro software and GraphPad Prism. The patch pipette solution consisted of (in mM): CsCl 110, $MgCl_2$ 4, HEPES 20, EGTA 10, GTP 0.35, adenosine triphosphate 4, creatine phosphate 14, pH 7.3, osmolarity approximately 310–315 mOsm. For experiments investigating Ca^{2+}-dependent inactivation the concentration of EGTA was reduced to 0.5mM. The extracellular solution was (in mM): 136 NaCl, 2 KCl, 1 $MgCl.6H_2O$, 10 Glucose, 10 HEPES, 5 CaCl, pH 7.3 osmolarity ~ 310 mOsm. For gabapentin treated cells, the drug was included in all extracellular solutions throughout the experiment.

Carbon fiber amperometry

Cells were placed into the recording bath and continuously washed with the same extracellular buffer used for calcium imaging and HPLC secretion experiments (see above). The carbon fiber amperometry electrode (Dagan Corporation, Minneapolis MN) was backfilled with 3 M KCl and positioned so that it just touched the surface of the cell. A potential of +700 mV was applied to the carbon fiber using a VA-10X amplifier (NPI Electronic GmbH, Tamm, Germany). Electrodes were changed frequently and control cells and gabapentin treated cells recorded alternately to minimize potential variability due to cell preparation, time in culture etc. Data were acquired using a 16-bit BNC-2090 analogue-to-digital converter (National Instruments, Austin, TX) and WinEDR acquisition software written by Dr. John Dempster, Ph.D. (Senior Lecturer, Strathclyde Institute for Pharmacy & Biomedical Sciences, University of Strathclyde, Glasgow, Scotland). Amperometric currents were filtered at 2 kHz and continuously sampled at 5 kHz. To evoke exocytosis, cells were stimulated by bath perfusion with 30 mM KCl. Amperometric spikes were detected and analyzed using a series of macros written by Dr. Eugene Mosharov, Ph.D. (see[26]) in IgorPro software (Wavemetrics Inc., Oswego OR). Events were detected using an amplitude threshold of 5 pA (which was 4–5 times the root mean squared (rms) noise of the baseline current), and were subsequently confirmed by visual inspection. All detected events were counted to assess overall secretory activity. However, to accurately analyze the individual

spike parameters (charge, amplitude, half width, slope) we excluded spikes that overlapped by > 10% and spikes with rise times > 10 ms that likely occurred distant from the carbon fiber and thus cannot be reliably collected. A median value for each spike parameter was calculated for each cell, and these values pooled and compared between control and gabapentin treated cells. Data collection and initial spike detection was performed in a blinded manner (*i.e.*, the experimenter was unaware of whether cells were from control or gabapentin treated groups).

Drugs and reagents

The AM-ester of Fura-2 was prepared as 1 mM stock in dimethyl sulfoxide and aliquots frozen for up to 1–2 weeks. The pentapotassium salt was prepared as an aqueous stock and stored at 4 °C. Gabapentin was prepared as a stock solution (100 – 300 mM) in sterile water and aliquots frozen. Carbachol and DMPP stocks were prepared in sterile water and kept refrigerated.

Statistical analyses

Unless otherwise noted all data are presented as mean ± SEM. All statistical analyses were performed using OriginPro software or GraphPad Prism software. Calcium imaging data were compared using one-way ANOVA with a Bonferroni multiple comparisons test. Catecholamine secretion detected using HPLC was compared between control and gabapentin treated cells using two-tailed, paired or independent Student's *t*-test as appropriate. Comparison of control and gabapentin treated cells at 0.1 and 1 mM were performed using a repeated measures ANOVA with Dunnett's multiple comparisons test. Peak I_{Ca} density was compared using using two-tailed, paired or independent Student's *t*-test as appropriate. To compare the inactivation time constants of I_{Ca}, current amplitude was normalized to peak and the decay from this peak was fit with a double exponential function using Prism5 software. The calculated fast and slow time constants generated by the fit were determined for each cell, and pooled values compared between control and gabapentin treated cells using a two-tailed independent t-test. For amperometry recordings the number of events followed a non-Gaussian distribution (determined using a Shapiro-Wilk normality test or D'Agostino & Pearson omnibus normality) so these data were analyzed using the nonparametric Mann-Whitney U-test. When analyzing individual spike characteristics (charge, amplitude, half width, and slope), if the parameters from all spikes in all cells are simply pooled then those cells with a high number of events will have greater weight than those cells with a low number of events. To avoid this we calculated median spike values for each cell which were then pooled and compared between control and gabapentin treated groups using two-tailed *t*-tests (for full discussion see [26]). In all cases data were considered to be significantly different if $p < 0.05$.

RESULTS

Gabapentin treatment reduces catecholamine release evoked by cholinergic agonists

In situ, chromaffin cells are innervated by cholinergic splanchnic nerve fibers. Activation of nicotinic acetylcholine receptors (nAChRs) on the chromaffin cells causes membrane depolarization, activation of voltage-gated Ca^{2+} channels, and influx of Ca^{2+} that triggers exocytosis of large dense core secretory granules. To mimic this cholinergic stimulation we used carbachol, an acetylcholine analogue that is resistant to cholinesterase activity. We wanted the timeline of gabapentin exposure in these cellular studies to broadly correlate with reported intra-and early postoperative clinical effects of preoperative gabapentin dosing. Thus, cells were incubated for 18 – 24 h in culture medium supplemented with 1 mM gabapentin, or an equivalent amount of vehicle (sterile water) for time matched controls. Cells seeded in 24-well tissue culture plates (~300,000 cells per well) were stimulated for 5

min with 100 μM carbachol and catecholamines were quantified using HPLC. As shown in figure 1A, basal secretion was not altered by gabapentin ($p = 0.15$). Carbachol evoked robust catecholamine secretion in control cells (11.5 ± 1.4 % of total content, n = 12, mean $\pm$ SEM) and there was a statistically significant reduction in gabapentin treated cells (8.9 ± 1.3 % of total content; n = 12; $p = 0.00023$). This decrease in carbachol-evoked release did not reflect altered catecholamine synthesis or storage because the total cellular content of epinephrine ($p = 0.16$) and norepinephrine ($p = 0.43$) was not altered in gabapentin treated cells (fig. 1B).

There is evidence for two populations of chromaffin cells that either express or lack phenylethanolamine N-methyltransferase, the enzyme that converts norepinephrine to epinephrine. Moreover, differential control of epinephrine and norepinephrine secretion has been reported [27,28]. However, this was not the case for gabapentin; epinephrine secretion was inhibited by 24 ± 3 % ($p = 0.0007$; n = 12, mean $\pm$ SEM) and norepinephrine secretion was inhibited by 23 ± 4 % ($p = 0.0001$; n =12, mean $\pm$ SEM) (fig. 1C). Carbachol activates both nicotinic and muscarinic receptors on chromaffin cells, but we found similar results when secretion was stimulated with a 5-min application of the selective nicotinic receptor agonist DMPP (30 μM) (fig. 1D). Gabapentin inhibited epinephrine secretion by 22 ± 4 % ($p = 0.042$; n = 4, mean $\pm$ SEM) and norepinephrine secretion by 16 ± 3 % ($p = 0.026$; n = 4, mean $\pm$ SEM), values that were not statistically different from those obtained using carbachol ($p = 0.85$ for norepinephrine and $p = 0.98$ for epinephrine).

To determine if carbachol evoked Ca^{2+} entry was altered by gabapentin we used fluorescent imaging of individual chromaffin cells loaded with Fura-2. Cells were continuously perfused with fresh extracellular recording solution and exposed to a 5-min application of carbachol (100 μM) to parallel the secretion studies outlined above. Carbachol elicited a sustained increase in intracellular $[Ca^{2+}]$ that reversed upon washout (fig. 1E). The magnitude of the Ca^{2+} elevation was not statistically different in cells treated with gabapentin compared to control cells (fig. 1F).

Gabapentin treatment reduces catecholamine release evoked by KCl, even though Ca^{2+} entry was increased

To bypass involvement of cholinergic receptors, we stimulated the cells with 30 mM KCl which directly depolarizes the membrane potential leading to Ca^{2+} influx through voltage-gated Ca^{2+} channels. Previous studies have shown that the secretory response to 30 mM KCl mimics that seen with acute stress [29]. There was a statistically significant reduction in KCl-evoked secretion from gabapentin treated cells compared to controls (fig. 2A) (p = 0.019; n = 8). There was a statistically significant inhibition of both epinephrine ($p = 0.002$; n = 8) and norepinephrine ($p = 0.006$; n = 8) secretion by gabapentin (fig. 2B). KCl elicited a robust increase in intracellular $[Ca^{2+}]$ that decayed by $\sim$ 40 % during the 5-min application (fig. 2C). The peak Ca^{2+} elevation (fig. 2D) and the Ca^{2+} elevation at the end of the 5-min stimulation (fig. 2D) were greater in gabapentin cells than in controls, and in both cases this difference was statistically significant ($p < 0.0001$). Thus, gabapentin reduced catecholamine release even though Ca^{2+} entry was modestly increased.

The inhibition of catecholamine release by gabapentin is concentration dependent

Clinically, gabapentin is given in a range of concentration and dosing paradigms. Plasma concentrations approach tens of micromolar and highly concentrative transport of gabapentin by the system L-amino acid transporter can occur in tissues or cells. Neutral amino acids including L-leucine and L-isoleucine competitively inhibit this transport and gabapentin binding to the $\alpha_2\delta$ subunit of Ca^{2+}-channels [30]. Each of these amino acids are present in tissue culture medium at $\sim$ 0.4 mM, (substantially higher than in plasma) so to overcome this competitive antagonism, we initially used 1mM gabapentin, consistent with

previous studies [14]. However, in other experiments we directly compared the ability of 0.1 mM and 1 mM gabapentin to inhibit catecholamine secretion evoked by KCl or carbachol. Both concentrations of gabapentin reduced catecholamine release compared to controls by a statistically significant amount ($p = 0.0002$, repeated measures ANOVA; $p < 0.05$ for 0.1 mM and $p < 0.001$ for 1mM using Dunnett's post test) (fig. 3A). Furthermore, the inhibition produced by 1 mM gabapentin (22 ± 3 %; n = 8, mean $\pm$ SEM) was statistically greater than that by 0.1 mM gabapentin (12 ± 3 %; n = 8, mean $\pm$ SEM) ($p = 0.039$). In figure 3B all data from the HPLC analyses of catecholamine secretion are combined to generate a concentration response curve which predicts an EC_{50} of 76 μM, close to relevant plasma concentrations in patients.

Gabapentin treatment does not alter the density or kinetics of voltage-gated Ca^{2+} channel currents

To enable more direct and precise insight into the effects of gabapentin on voltage-gated Ca^{2+} channel currents (I_{Ca}) we used whole-cell patch clamp electrophysiology. We have previously shown that chromaffin cells express predominantly N-type, and P/Q-type calcium channels with a smaller (10–15 %) contribution from L-type channels [31,32]. Acute application of gabapentin or pregabalin has been reported to inhibit I_{Ca} in some studies [9–12] but not in others [13–15], and has never been tested in chromaffin cells. Therefore, we first investigated the effects of acute gabapentin application on I_{Ca}. Chromaffin cells were voltage-clamped at −80 mV and I_{Ca} was elicited by a 20 ms step depolarization to +10 mV once every 20 s. Bath perfusion of gabapentin (1 mM) for 10-minutes had no obvious effect on I_{Ca} amplitude (fig. 4A). The gradual rundown in current amplitude during the course of the experiment (12 ± 4 % decline; n = 6, mean $\pm$ SEM) was not significantly different ($p = 0.68$) than in control cells (15 ± 4 %; n = 7; mean $\pm$ SEM; not shown).

Next, to parallel the secretion studies outlined above (fig. 1–3), we compared I_{Ca} in control cells and cells treated for 18–24 h with 1 mM gabapentin. The peak current density, calculated by normalizing peak current amplitude to membrane capacitance (cell size), was not significantly different in gabapentin treated cells compared to matched controls (fig. 4B; $p = 0.92$). There was also no discernible shift in the current-voltage relationship of I_{Ca} (fig. 4C). Voltage-gated Ca^{2+} channels are subject to both voltage-dependent inactivation and Ca^{2+}-dependent inactivation [33–37] that control the amount and timing of Ca^{2+} entry during sustained or repetitive stimulation. Pregabalin disrupts inactivation of I_{Ca} in Calyx of Held neurons [9], so we investigated if gabapentin altered inactivation of I_{Ca} in chromaffin cells. For these experiments we lowered the concentration of EGTA in the patch-pipette solution from 10 mM to 0.5 mM because Ca^{2+}-dependent inactivation of N- and P/Q-type calcium channels is blocked by higher concentrations of EGTA or other Ca^{2+} chelators [37,38]. Cells were stimulated with a 500 ms step depolarization from −80 mV to +10 mV (fig. 4D) to elicit an inward I_{Ca}. The peak density of I_{Ca} was slightly smaller in cells treated with gabapentin (1 mM, 18–24 h) than in controls, but this difference was not statistically significant (54 ± 4.6 pA/pF, n = 10 compared to 64 ± 4.4 pA/pF, n = 8, mean $\pm$ SEM, $p = 0.11$). The extent of inactivation at the end of the 500 ms step (% decay from peak amplitude) was not significantly altered in gabapentin treated cells (66 ± 3 %, n = 10, mean $\pm$ SEM) compared to control cells (74 ± 4 %, n = 8, mean $\pm$ SEM; $p = 0.13$). The inactivation of I_{Ca} was well fit with a double exponential decay and we found no statistically significant difference in the fast or slow time constants derived from these fits (fig. 4E). We repeated the same experiment but with 10 mM EGTA in the patch pipette solution which blocks Ca^{2+}-dependent inactivation but leaves voltage-dependent inactivation intact. Gabapentin had no effect on this voltage-dependent inactivation (32 ± 2 % n = 7 in controls compared to 33 ± 2 % n = 6 in gabapentin treated cells; $p = 0.82$, mean $\pm$ SEM).

We also tested whether G protein mediated inhibition of I_{Ca} was altered by gabapentin. We and others have shown previously that adenosine triphosphate coreleased with catecholamines mediates feedback inhibition of I_{Ca} and thus transmitter release in chromaffin cells [21]. This inhibition is due to activation of P2Y autoreceptors and downstream G proteins which directly inhibit the channels [21]. Gabapentin treatment did not alter this important regulatory pathway; application of adenosine triphosphate (100 μM) inhibited I_{Ca} in control cells by 48 ± 7 % (n = 6, mean ± SEM) and in gabapentin treated cells by 49 ± 3 % (n = 9, mean ± SEM) ($p = 0.98$ comparing control to gabapentin treated cells). Thus, we found no statistically significant difference in the density, kinetics, or regulation of I_{Ca} in gabapentin treated cells compared to controls.

Gabapentin reduces the number but not the quantal size or kinetics of secretory vesicle fusion events

The overall reduction in catecholamine release that we report could be due to fewer vesicles fusing with the plasma membrane and/or altered content or release of catecholamine from each vesicle. Hydralazine and some beta-blockers have been reported to accumulate in secretory vesicles of chromaffin cells and thereby displace catecholamines leading to reduced vesicular content and altered release kinetics [39,40]. Therefore, to assess these possibilities and gain more precise mechanistic insight we used carbon fiber amperometry. Briefly, a carbon fiber electrode was positioned immediately adjacent to an individual chromaffin cell (fig. 5A). A potential of +700 mV was applied to the fiber such that catecholamines released from the adjacent cell were rapidly oxidized generating two electrons for each molecule of catecholamine. Because of the sensitivity and precise spatial and temporal resolution, this approach can readily resolve catecholamine release from individual vesicles fusing with the plasma membrane. Each fusion event leads to a transient current "spike" (fig. 5A). These spikes can be counted as a measure of the number of vesicles that undergo exocytosis. We compared secretion evoked by 30 mM KCl and found a statistically significant reduction (~35 %) in the rate of fusion events in gabapentin treated cells compared to control cells (fig. 5B).

We also analyzed the individual amperometric spikes in control and gabapentin treated cells. The charge (integral) of each spike is directly proportional to the number of catecholamine molecules released from a single vesicle. The amplitude of the spikes reflects the peak concentration of catecholamine at the electrode tip whereas the "half-width" and slope reflect the duration and speed of catecholamine release respectively. These characteristics were determined for each spike and a median value calculated for each cell. These median spike parameters were then pooled and we found no statistically significant differences between control and gabapentin treated cells (fig. 5C–F) ($p > 0.63$ for all comparisons). Thus our data reveal that gabapentin reduced the number of vesicles that underwent exocytosis, but did not alter the amount or kinetics of catecholamine released from those vesicles that did fuse with the plasma membrane.

DISCUSSION

To our knowledge this is the first study to investigate the effects of gabapentin on stimulus-secretion coupling in adrenal chromaffin cells. Our rationale for doing so was motivated by two primary goals. First, chromaffin cells are widely used as a model to investigate regulation of Ca^{2+}-channels and neurosecretion [20–22]. Thus, we reasoned this would be a good system in which to evaluate the cellular mechanisms of gabapentin. Second, catecholamines, endogenous opioids [23], and other hormones released from chromaffin cells play central roles in the sympathoadrenal stress response, and might contribute to stress-related enhancement of mechanical hyperalgesia [24]. Therefore, we postulated that altered

adrenal catecholamine release might contribute to hemodynamic stabilization and perhaps other beneficial perioperative effects of gabapentin.

Gabapentin reduces catecholamine release from adrenal chromaffin cells

Gabapentin has been reported to block elevated serum levels of norepinephrine produced by spinal nerve ligation [41], or conversely to activate the descending noradrenergic system and increase norepinephrine release in the spinal cord, likely through modulation of glutamatergic signaling in the locus coeruleus [42–45]. Thus, the effects of gabapentin on catecholamine levels *in vivo* likely involve central and peripheral sites of action, including indirect effects through altered network excitability and direct targeting of neurosecretory cells and mechanisms. However, the effects of gabapentin on adrenal chromaffin cells, a primary source for circulating catecholamines, was unknown prior to this study. To mirror the relevant time-frame for intra-and early postoperative effects of preoperative gabapentin dosing, we treated cells with gabapentin for 18–24 h and compared catecholamine release to matched controls. We show that gabapentin did not alter the catecholamine content of the chromaffin cells, but produced a statistically significant reduction in secretion evoked by cholinergic agonists or by direct membrane depolarization (30 mM KCl) (fig. 1–3). The modest extent of this inhibition (~ 20–25 %) is in line with previous reports investigating neurotransmitter release in brain slices [44,46], and expected for a drug that is generally well tolerated. Thus, we demonstrate for the first time that gabapentin has direct actions on chromaffin cells that might contribute to modulation of the sympathoadrenal stress response. It will be of interest in future studies to determine if gabapentin also has indirect effects by altering sympathetic drive to the adrenal medulla.

The inhibition of catecholamine release was not mediated by altered calcium entry

Currently, the consensus view is that gabapentin and pregabalin target the $\alpha_2\delta_1$ or $\alpha_2\delta_2$ subunit of voltage-gated Ca^{2+} channels to mediate many of their physiological effects (for review see [8]). Calcium influx through voltage-gated Ca^{2+} channels triggers neurotransmitter and hormone release leading to the hypothesis that gabapentin reduced transmitter release by inhibiting Ca^{2+} entry. Indeed, some studies reported acute inhibition of I_{Ca} by gabapentin or pregabalin [9–12], although others found no effect [13–15]. Here we report that acute (10-min) gabapentin application had no effect on I_{Ca} in chromaffin cells (fig. 4A).

Of more relevance to our investigations of catecholamine secretion, previous studies showed that longer-term (17–48 h) incubation with gabapentin / pregabalin reduced the amplitude of I_{Ca} due to a decrease in the number of channels at the plasma membrane [13,14,16]. This was dependent on the $\alpha_2\delta$ subunit, but appeared to involve an intracellular action of gabapentin to disrupt trafficking / recycling of the channels from endosomal compartments to the plasma membrane [17]. Using Fura-2 imaging, we found no effect of gabapentin (18–24 h) on the intracellular $[Ca^{2+}]$ elevation produced by cholinergic agonists (fig. 1), while the $[Ca^{2+}]$ elevation evoked by 30 mM KCl was slightly increased (fig. 2). We used patch-clamp electrophysiology and found no statistically significant differences in I_{Ca} density or kinetics in gabapentin treated cells (18–24 h incubation) compared to controls (fig. 4). We also found no statistically significant alteration in regulation of I_{Ca} by endogenous P2Y purinergic autoreceptors, an important mechanism that mediates feedback inhibition of catecholamine secretion [21]. These data suggest the enhanced calcium entry seen with KCl stimulation was not due to direct effects of gabapentin on I_{Ca}. Pregabalin was previously reported to enhance Ca^{2+} entry in a subset of sensory neurons perhaps through modulation of K^+ channels [47], but we did not investigate this possibility in the current study.

It remains unclear why gabapentin had no effect on I_{Ca} in chromaffin cells, but it is possible that longer incubations are required to observe the trafficking effect. Alternatively, specific

Ca^{2+} channel subunit combinations or splice variants might be required [13], and not highly expressed in chromaffin cells. Regardless of the reason, our data provide direct evidence that gabapentin can inhibit catecholamine secretion independently from effects on Ca^{2+} entry, perhaps through disruption of the exocytotic machinery or vesicular trafficking events.

Gabapentin reduced the number but not the quantal size of secretory vesicle release events

In general, an overall reduction in transmitter release could reflect fusion of fewer secretory vesicles with the plasma membrane and/or alterations in the amount or kinetics of transmitter release from each vesicle. One advantage to using chromaffin cells is the ability to directly detect and quantify catecholamine release from individual vesicles using carbon fiber amperometry. Previous work demonstrated that second messenger pathways can alter the content and/or emptying of secretory vesicles upon stimulation[48–53]. Furthermore, hydralazine and β blockers can accumulate in secretory vesicles and thereby reduce the amount and kinetics of catecholamine release from each vesicle[39,40]. Using carbon fiber amperometry we show that gabapentin reduced the number of vesicles that undergo exocytosis, but did not change the amount or kinetics of catecholamine released from these unitary events (fig. 5).

Taken together, our data suggest that Ca^{2+} entry is unaltered by gabapentin but is less effective at triggering vesicle fusion. Consistent with this, gabapentin / pregabalin inhibited spontaneous synaptic release events or exocytosis evoked by hypertonic sucrose, even though these events are thought to occur independent from Ca^{2+} entry [18,19]. It is possible that binding to the $\alpha_2\delta$ subunit might allosterically disrupt coupling of Ca^{2+} channels with the exocytotic machinery [18]. Gabapentin and pregabalin are also substrates for the neutral L-amino acid transporter so may act intracellularly. Recently, $\alpha_2\delta$ subunits were identified on neuropeptide containing large dense core secretory vesicles isolated from dorsal spinal cord neurons [54]. Perhaps analogous to the disruption of channel recycling from endosomal compartments [17], gabapentin might alter secretory vesicle trafficking, docking, or priming, and thus control the number of fusion competent vesicles at the plasma membrane.

Relating cellular mechanism to perioperative physiological effects

There is increasing interest in potentially beneficial effects of acute (up to 24 h) preoperative gabapentin [1]. While much of this work has focused on analgesia, several reports have demonstrated that gabapentin blunted the hemodynamic response to direct laryngoscopy / tracheal intubation [2,3], or skull pin insertion[55]. Although the mechanism was not directly addressed in these studies, these procedures are known to elicit an acute stress response including increased levels of circulating catecholamines [4–7]. This led us to postulate that gabapentin might act in part through reducing sympathoadrenal stress hormone release. Consistent with this hypothesis, we provide the first demonstration that gabapentin inhibits catecholamine release from isolated adrenal chromaffin cells. As already mentioned, gabapentin exerts widespread actions, perhaps including altered neuronal excitability and sympathetic drive to the adrenal medulla in addition to the direct effects on chromaffin cells reported here. Thus, further *in vivo* studies are required to corroborate our findings, but careful attention needs to be paid to the anesthetic regimen that might in itself alter the hemodynamic response and/or sympathoadrenal hormone release [4,56]. Previous work has shown that opioids [31,48] and anesthetics including etomidate, propofol, and isoflurane can modulate calcium signaling and transmitter release in chromaffin cells [57–60]. Given the perioperative exposure to these classes of drug, it is pertinent to determine if, and how, they impact regulation of catecholamine release by gabapentin. Other mechanisms such as altered neuronal excitability, vagal reflexes, or direct effects on the cardiovascular system might also contribute to the hemodynamic stabilization produced by gabapentin. Pregabalin

inhibits L-type Ca^{2+} channels in smooth muscle cells isolated from cerebrovascular arteries [61], but gabapentin does not alter I_{Ca} in cardiac myocytes [62]. Generally, gabapentin is well tolerated and thought to have little effect on cardiovascular function under resting conditions, consistent with the idea that perioperative hemodynamic stabilization involves blunting of the acute stress response.

Surgery is well known to elicit a stress response that can persist for hours or days postoperatively. It is tempting to speculate that by reducing sympathoadrenal stress hormone release gabapentin might impact a variety of postoperative physiological parameters including hemodynamics and inflammation. This might relate to some of the antihyperalgesic effects of gabapentin because chronic elevation of epinephrine and peripheral nerve catecholamine chemosensitivity have been implicated in the induction and maintenance of selected forms of mechanical hyperalgesia and neuropathic pain[24,63–65]. Although more studies are needed, this paper identifies altered adrenal catecholamine release as a feasible mechanism underlying some of the perioperative effects of gabapentin. Our work also demonstrates the utility of adrenal chromaffin cells as a model system to dissect the cellular mechanism(s) of this widely prescribed drug.

Acknowledgments

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Summary Statement

What we already know about this topic

- Gabapentin has acute preemptive analgesic effects and blunts the hemodynamic response to laryngoscopy and tracheal intubation.

- Gabapentin binds with high affinity to a subunit of voltage-gated calcium channels.

- Calcium influx triggers secretory vesicle exocytosis.

What this article tells us that is new

- Gabapentin reduced catecholamine release from adrenal chromaffin cells without altering their catecholamine content.

- Calcium entry was unaltered by gabapentin but calcium was less effective in triggering vesicle fusion.
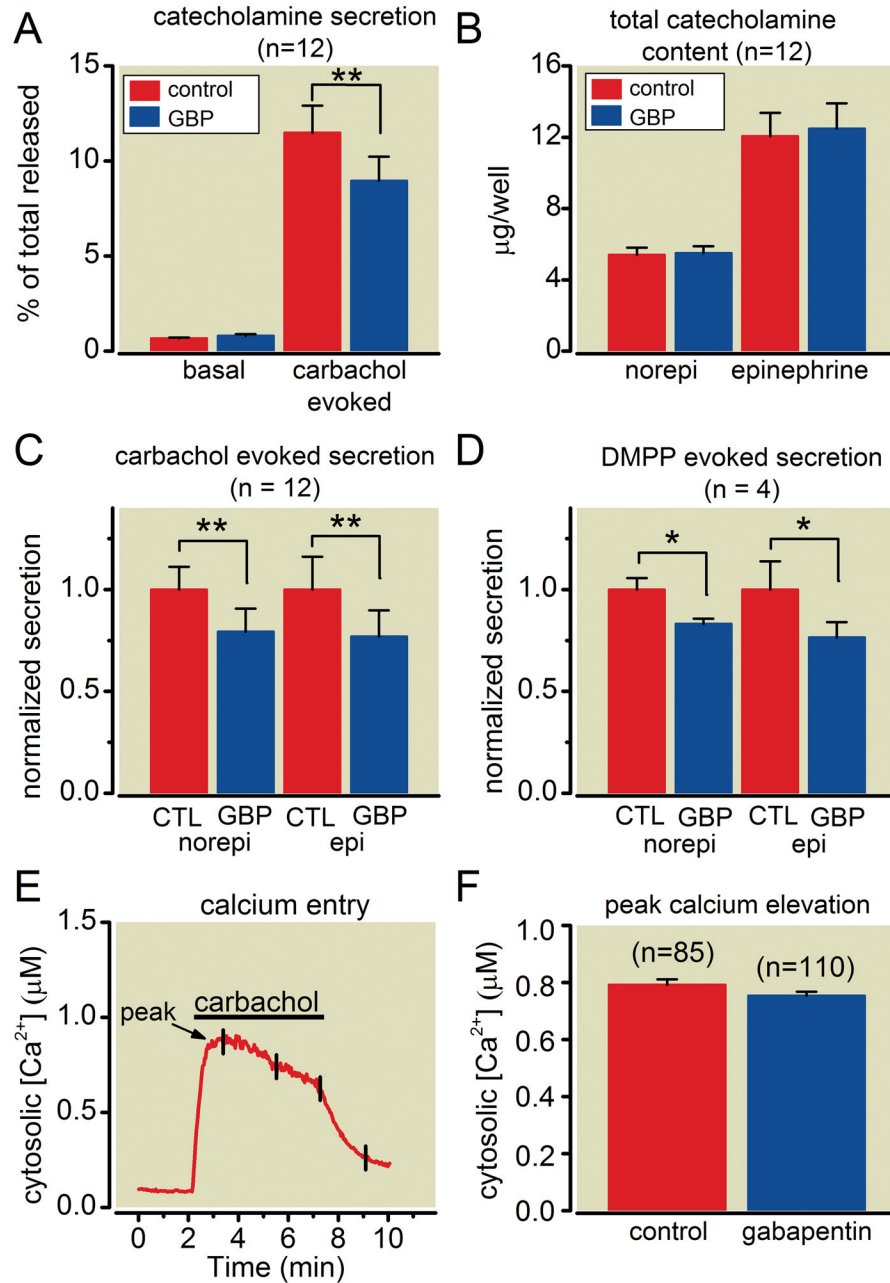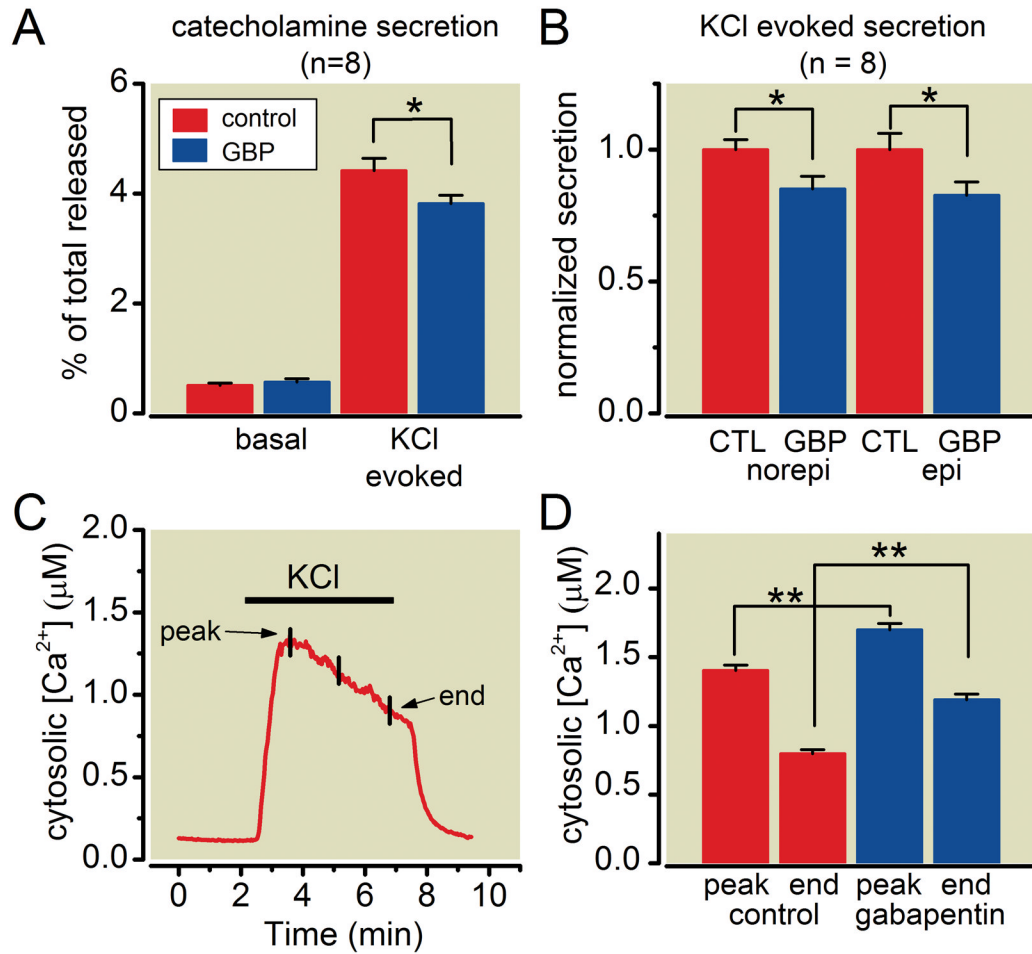
Figure 1. Gabapentin reduces catecholamine secretion but not calcium entry evoked by cholinergic stimulation

(A) Chromaffin cells were seeded on 24-well plates and treated with 1mM gabapentin (GBP) or vehicle (control) for 18–24 h. The amount of catecholamines released under basal conditions (in the absence of secretagogue) or during a 5-min stimulation with carbachol (100 μM) was determined using high performance liquid chromatography (HPLC) and expressed as a percentage of total cellular content (mean ± SEM). Gabapentin significantly reduced carbachol-evoked secretion (** $p = 0.00023$) but not basal release ($p = 0.15$). **(B)** Gabapentin treatment (GBP) did not alter the total cellular content of norepinephrine ($p = 0.43$) or epinephrine ($p = 0.16$) compared to control cells (mean ± SEM). **(C)** Secretion evoked by carbachol in gabapentin treated cells (GBP) was normalized to controls (CTL).

Gabapentin reduced both epinephrine (epi) ($p = 0.0007$) and norepinephrine (norepi) ($p = 0.0001$) secretion to a similar extent (mean ± SEM). **(D)** Same layout as in panel C except using a 5-min stimulation with 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) (30 µM), a selective nicotinic receptor agonist. Gabapentin significantly reduced both epinephrine (epi) ($p = 0.042$) and norepinephrine (norepi) ($p = 0.026$) secretion. **(E)** Representative experiment showing the $[Ca^{2+}]_i$ increase evoked by a 5-min application of 100µM carbachol in FURA-2 loaded chromaffin cells (n = 8 cells, mean ± SEM - for clarity error bars are only shown for a few data points). **(F)** Pooled data from multiple experiments like that shown in panel E. The peak increase in $[Ca^{2+}]_i$ evoked by carbachol was not significantly altered in gabapentin treated cells compared to control cells (mean ± SEM; p = 0.10).

Figure 2. Gabapentin reduces KCl-evoked catecholamine secretion even though calcium entry was increased

(A) Chromaffin cells were seeded on 24-well plates and treated with 1mM gabapentin (GBP) or vehicle (control) for 18–24 h. The amount of catecholamines released under basal conditions (in the absence of secretagogue) or during a 5-min stimulation with 30 mM KCl was determined using high performance liquid chromatography and expressed as a percentage of total cellular content (mean ± SEM). Gabapentin treatment significantly reduced KCl-evoked secretion (* $p < 0.019$; n = 8) but not basal release ($p = 0.42$; n = 8). **(B)** Secretion evoked by KCl in gabapentin treated cells (GBP) was normalized to controls from the same plate (mean ± SEM). There was a statistically significant inhibition of both epinephrine ($p = 0.002$; n = 8) and norepinephrine ($p = 0.006$; n = 8) secretion by gabapentin. **(C)** Representative experiment showing the $[Ca^{2+}]_i$ increase evoked by a 5-min application of 30mM KCl in FURA-2 loaded chromaffin cells (n = 18 cells, mean ± SEM - for clarity error bars are only shown for a few data points). **(D)** Pooled data from multiple experiments like that shown in panel C. The peak increase in $[Ca^{2+}]_i$ (peak) and the sustained increase at the end of the KCl application (end) were significantly greater in gabapentin treated cells (n = 97) compared to controls (mean ± SEM; n = 128) (** $p < 0.0001$).
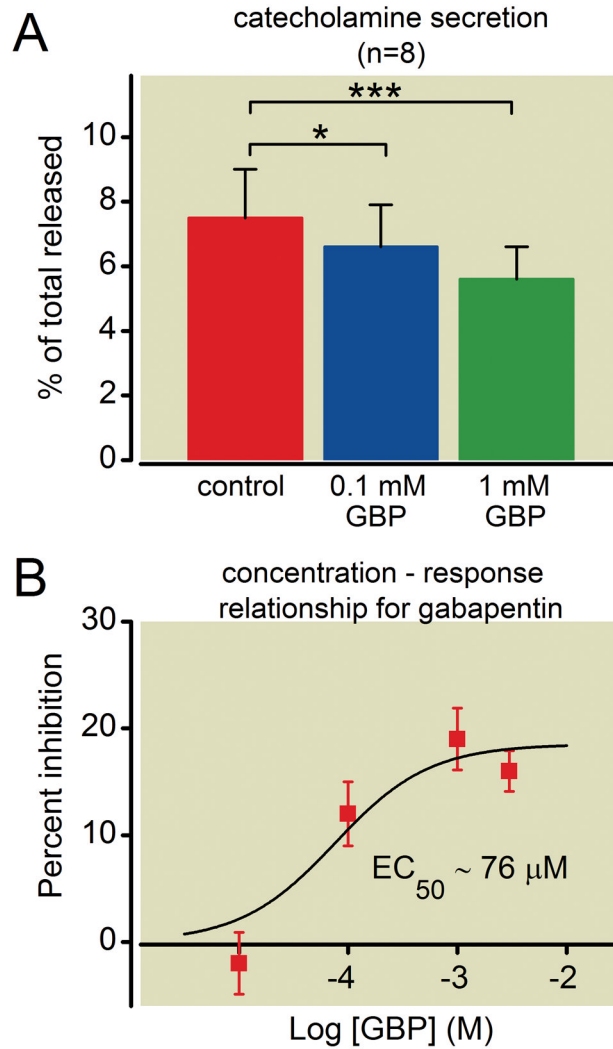
Figure 3. The inhibition of catecholamine secretion by gabapentin is concentration-dependent
(**A**) Chromaffin cells were seeded on 24-well plates and treated with vehicle (control) or gabapentin (0.1mM GBP or 1mM GBP) for 18–24 h. Evoked catecholamine secretion was determined using high performance liquid chromatography (HPLC) and expressed as a percentage of total cellular content (mean ± SEM). Both concentrations of gabapentin significantly reduced secretion compared to controls (n = 8; * $p < 0.05$; *** $p < 0.001$). (**B**) Data from all HPLC experiments were pooled and percent inhibition of evoked catecholamine secretion (mean ± SEM) plotted against $\log_{10}$ of gabapentin concentration (10 μM, n = 4; 100 μM, n = 8; 1mM, n = 20; 3 mM, n = 4). A concentration response curve was generated by fitting the data to a Boltzmann function with a Hill slope = 1, and yielded an estimated EC_{50} of 76 μM.
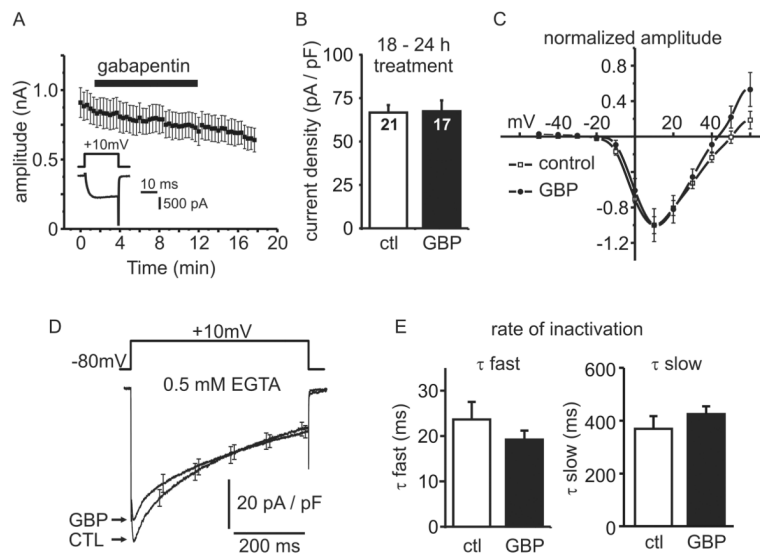
Figure 4. Gabapentin does not alter the density or kinetics of voltage-gated Ca^{2+} channel currents

(**A**) Whole cell patch-clamp electrophysiology was used to record I_{Ca} elicited by 20 ms step depolarizations (see inset), and the peak amplitude (mean ± SEM) plotted against time. Acute application of 1mM gabapentin (indicated by black bar) had no effect on I_{Ca} amplitude. (**B**) Cells were treated with vehicle (ctl) or 1mM gabapentin (GBP) for 18–24 h prior to recording. Peak I_{Ca} was normalized to cell size (membrane capacitance) to yield current density. There was no change in current density (mean ± SEM) in gabapentin treated cells (n = 17) compared to control cells (n = 21) ($p = 0.92$). (**C**) The current-voltage relationship for I_{Ca} was not shifted by treatment with 1mM gabapentin for 18 – 24 h. I_{Ca} amplitude (mean ± SEM) was normalized to the peak inward current in each cell to facilitate comparison of the voltage-dependence. (**D**) Inactivation of I_{Ca} during a 500 ms step depolarization in control cells and cells treated for 18– 24 h with 1mM gabapentin. Traces represent the data from 8 control cells and 10 gabapentin treated cells (mean ± SEM). For clarity, error bars are shown for only a few data points. (**E**) Inactivation of I_{Ca} during a 500 ms step was fit with a double exponential decay and the time constants from each cell were pooled. There was no difference in either the fast (τ fast) ($p = 0.28$) or slow (τ slow) ($p = 0.31$) inactivation time constants between control (n = 8) and gabapentin treated cells (n = 10) (mean ± SEM).
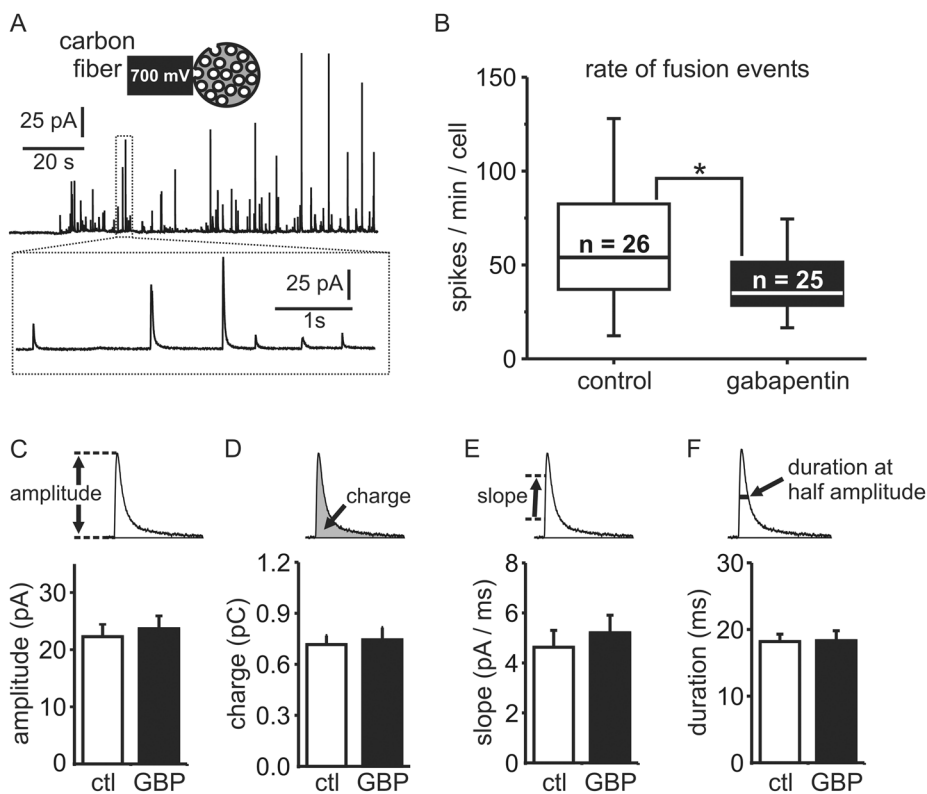
Figure 5. Gabapentin reduces the number of secretory vesicle fusion events but not the quantal size or kinetics of catecholamine release from each vesicle

Carbon fiber amperometry was used to quantify the number, quantal size, and kinetics of individual vesicular fusion events. **(A)** A representative amperometry recording from a control cell stimulated with 30 mM KCl. Each upward deflection (spike) on the current trace is produced by oxidation of the catecholamines released from a single vesicular fusion event. The inset shows a cartoon representation of the recoding configuration (above) and an expanded view of a few spikes is shown below. **(B)** The number of amperometric spikes was determined for each cell over a 2-min period. The box graph shows the 25th percentile, median, and 75th percentile distribution of vesicular fusion rate (spikes per minute) for control cells (n = 26) and cells treated for 18–24 hours with 1mM gabapentin (n = 25). The whiskers represent the smallest and largest non-outliers in each population of cells. Gabapentin produced a statistically significantly reduction in the rate of fusion events compared to matched controls (* $p = 0.046$, Mann-Whitney test). **(C–F)** Other parameters of the amperometric spikes (amplitude, charge, slope, and duration) were analyzed and a median value for each cell calculated. Pooled values (mean ± SEM) for each parameter are shown and were compared. No statistically significant differences were found between gabapentin treated cells (GBP) and control cells (ctl).